Glycoconjugate Journal 16, 99–107 (1999)

Filamentous fungi as production organisms for glycoproteins of bio-medical interest

Marleen Maras[1], Irma van Die[2], Roland Contreras[1], and Cees A.M.J.J. van den Hondel[3]*

[1]*Department of Molecular Biology, Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium*
[2]*Department of Medical Chemistry, Vrije Universiteit, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands*
[3]*Department of Molecular Genetics and Genetechnology, TNO Nutrition and Food Research Institute, P.O. Box 360, 3700 AJ, Zeist, The Netherlands*

Filamentous fungi are commonly used in the fermentation industry for large scale production of glycoproteins. Several of these proteins can be produced in concentrations up to 20–40 g per litre. The production of heterologous glycoproteins is at least one or two orders of magnitude lower but research is in progress to increase the production levels. In the past years the structure of protein-linked carbohydrates of a number of fungal proteins has been elucidated, showing the presence of oligo-mannosidic and high-mannose chains, sometimes with typical fungal modifications. A start has been made to engineer the glycosylation pathway in filamentous fungi to obtain strains that show a more mammalian-like type of glycosylation. This mini review aims to cover the current knowledge of glycosylation in filamentous fungi, and to show the possibilities to produce glycoproteins with these organisms with a more mammalian-like type of glycosylation for research purposes or pharmaceutical applications

Keywords: **filamentous fungi, homologous and heterologous protein secretion, N-glycosylation, O-glycosylation, phosphorylation, protein-linked carbohydrates.**

Introduction

The expression of human genes in a variety of heterologous expression systems has become an important technique to produce recombinant proteins for research purposes or pharmaceutical applications. It is generally recognised that there is no universal expression system available for (biopharmaceutical) production, and the selection of a cell type for expression of heterologous proteins depends on a number of criteria. One of the important criteria to be considered, is whether a protein needs to be (correctly) glycosylated for its application. Many human therapeutics are glycoproteins, and it is known that the glycosylation modulates numerous protein characteristics [1,2]. Glycoproteins that are not correctly glycosylated may be misfolded, biologically inactive, cleared from the circulation too fast, or exert an unwanted immunological response. Chinese hamster ovarian cell lines (CHO cells) have been shown to be suitable hosts for the expression of a number of sialylated glycoproteins (see for example [3,4]). The availability of several mutant CHO cell lines, stable transfected with additional glycosyltransferases, increases the potential of this system to "mimic" the natural glycosylation of specific glycoproteins.

For a number of glycoproteins, the presence of site-specific N-glycosylation is important, but the precise structure of the glycans apparently does not directly influence the biological function of the protein. Dependent on the application of the glycoprotein, it may be considered to use alternative eukaryotic expression systems for the production of such glycoproteins, e.g., insect cells, plants or filamentous fungi. Insect cells infected with recombinant baculovirus vectors already have become a popular expression system for the production of mammalian glycoproteins [5]. Recombinant glycoproteins expressed in this system mostly contain truncated paucimannosidic N-glycans [5]. In some cases the presence of complex-type glycans has been reported, both "wanted" glycans containing a LacNAc unit, and "unwanted" glycans containing the immunogenic core α 3-fucose epitope [6–9].

Filamentous fungi are natural secretors of a number of glycoproteins (mainly enzymes), often in abundant quantities, and are able to grow on relatively inexpensive media. Due to these properties and also to their GRAS (General Regarded As Safe) status the filamentous-fungal species *Aspergillus niger, Aspergillus awamori* and *Aspergillus oryzae* are widely used by the fermentation industry for the

*To whom correspondence should be addressed. Fax: 31-695 72 24

production of a great variety of extracellular enzymes mainly used in the food industry [10]. Also the filamentous fungus *Trichoderma reseei* is widely used for the production of enzymes used in the laundry and paper and pulp industry. Initially the production levels of these enzymes were relatively low, but by performing long-lasting classical strain-improvement programs, industrial production strains have been obtained by which high levels of fungal-enzyme (e.g., more than 20 g/l of *A. niger* glucoamylase and more than 40 g/l of *T. reesei* cellobiohydrolase I) are produced [11].

The application of modern molecular-genetic techniques has, however, greatly accelerated fungal strain improvement and has created new opportunities for the production of fungal enzymes, but also of non-fungal enzymes and pharmaceutical or diagnostic proteins. In the last years all the "tools" have been developed, which are required for (molecular)-genetic analysis of *A. niger, A. awamori, A. oryzae* and *T. reesei* and for construction of high production strains [12].

Filamentous fungi as host for the expression of heterologous proteins

To evaluate the potential of filamentous fungi to high level production of fungal and non-fungal glycoproteins by genetic modification, the overproduction of filamentous-fungal enzymes by *A.niger, A. oryzae* and *T. reesei* has been studied. The most common strategies by which high level production of fungal enzymes have been achieved are based on the use of well characterised strong, constitutive or regulated gene expression signals and the generation of multicopy strains. Such strains can be obtained using cosmid vectors, containing several gene-copies, and/or using selection markers which facilitate multicopy vector-integration. With these systems at least 10–20-fold improvement of most fungal enzymes could be achieved, resulting in the production of up to 1–2 gr/l in a shake flask culture (for a review see [12]). From these overexpression studies it also became clear that a limitation at the level of transcription is the main bottleneck for achieving higher production levels.

For the production of heterologous proteins of mammalian, bacterial, avian or plant origin, the expression systems that have been developed for the overproduction of fungal proteins, have been used. Unfortunately the initial production levels were much lower and with some exceptions reached levels that did not exceed a few milligrams per litre (see reviews [13–15]). Several empirical improvement studies and a limited number of systematic analysis-studies have provided evidence that the following factors negatively influence the initial production levels: i) mRNA-instability [16], ii) codon usage [17], iiii) inefficient translocation, folding and transport through the secretion pathway [18,19] and iii) extracellular degradation [20–22]. From the data available, it is clear that the limiting factors often depend on the protein to be expressed and/or the filamentous fungal host strain. On the basis of the identified factors that limited the efficient production of non-fungal proteins, several strategies have been developed for improved production (see reviews [19,23]). Most of these strategies are similar to those for fungal proteins [12] and comprise (i) the introduction of large number of copies, (ii) the use of efficient transcription, translation and secretion control-signals, (iii) the construction and use of protease-deficient host strains, (iv) development of optimal production media and (v) gene fusion with a gene encoding part or whole of a well-secreted protein. Especially the latter strategy has shown to be very effective resulting in an improvement varying from 5 to 1000-fold, depending on the protein, resulting in protein levels varying from 5 mg/l to 250 mg/l (see Table 1). Even higher production levels, up to 1–2 g/l, were obtained for the glycoproteins bovine chymosine [24] and lactoferrin [25] when high-level production strains were subjected to several rounds of mutagenesis and selection. Research is still in progress to further improve the production of glycoproteins by optimising the primary sequence of the genes of interest, improvement of the mRNA stability, overproduction of chaperones and foldases, and isolation of strains which are deficient in protease acting during the secretion process, in the vacuole and/or in the cultivation medium.

The structures of protein-linked carbohydrates from filamentous fungi

Similar as mammalian cells, filamentous fungi produce soluble and membrane-bound post-translationally modified proteins, that may carry N- and/or O-glycans. Filamentous fungi most often synthesise small high-mannose type N- and O-glycans [23]. Table 2 gives an overview of characterised glycans from several species of filamentous fungi. This overview shows a remarkable complexity in glycan structures. At the moment it is not clear which enzymes contribute to the formation of these oligosaccharides. Probably, a whole set of glycosyltransferases and glycosidases in the secretion apparatus are involved. In addition, filamentous fungi are known to secrete different glycosidases to the extracellular medium, that may trim protein-linked glycans [26–31]. The latter modifications may add to the observed heterogeneity of the glycans.

Sofar, no typical mammalian-like complex-type glycans have been found in filamentous fungi [32]. Some of the glycans found (see Table 2) resemble mammalian high-mannose glycans [Man(6-9)GlcNAc2]. In addition, typical "fungal-type" glycans have been identified on different *Aspergillus* and glycoproteins that are structurally different from the mammalian glycans [Man(5–12)GlcNAc2] and that are probably synthesised by not yet characterised fun-

Table 1. Production of heterologous proteins by filamentous fungi using a gene-fusion strategy

Protein and host	*"Carrier" gene*	*"Kex2" linker*[a]	*Protein level*	*Improvement factor*	*Ref.*
Bovine chymosine					
A. awamori	–	–	8 mg/l		[25]
	A. awamori glaA 1-614		140 mg/l	10–20 ×	[25]
Fab-antibody fragments					
T. reesei	–	–	0.3–1 mg/l		[82]
	T. reesei cbhI 1-466		5–40 mg/l	5–100	[82]
Hen egg-white lysozyme					
A. niger	–	–	50 mg/l		[13]
	A. niger glaA 1-498	+	1000 mg/l	>20 ×	[13]
Human Interleukin-6					
A.niger	–	–	nd[b]		[20]
	A. niger glaA 1–514	+	15 mg/l	>1000 ×	[20]
A. awamori	–		50 ug/l		[17]
	A. niger glaA 1-514		15 mg/l	300 ×	[17]
Human lactoferrin					
A. awamori	+	+	25 mg/l		[25]
	A. niger glaA 1–498	+	>250 mg/l	>10–50 ×	[25]
Single chain Fv anti-lysozyme					
A. niger	–	–	10–30 mg/l		[83]
	A. niger glaA 1-514	+	50–80 mg/l	2–6 ×	[83]
Human thrombomodulin					
A. chrysogenum	–	–	15 mg/l		[84]

[a]Cleavage occurs by a KEX2-like protease for which a recognition site had been introduced in the fusion protein; [b]nd: not detectable

gal mannosyltransferases in the Golgi compartment. Very small N-glycans such as Man(α1–2)GlcNAc2 were also found, for instance on *A. awamori* glucoamylase [33]. The genes of specific α-1,2-mannosidases from *T. reesei, A. saitoi* and *P. citrinum* have been cloned [34,35; Maras et al., submitted manuscript], but it is not clear whether these enzymes are actually involved in processing of N- and/or O-glycans.

In contrast to *Saccharomyces cerevisiae,* hyperglycosylation is not a typical feature for filamentous fungi. Hyperglysolation of proteins, however, has been detected in some strains of filamentous fungi [36] and it may be dependent on the growth conditions if an heterologous protein contains small-sized N-glycans or hyperglycosyl structures [37].

Recently, the presence of single, non-substituted GlcNAc residues has been detected on potential N-glycosylation sites of proteins derived from certain fungal species, such as *T. reesei* and *Aspergillus tubigensis* [38–40]. Until now the biosynthesis of this type of glycosylation is not clear. A deglycosylating enzyme, analogous to 'Endo H' could be responsible for the formation of these single N-acetylglucosamines, or the single GlcNAc could result from extensive trimming by several different exoglycosidases. Alternatively, transfer of GlcNAc to potential N-glycosylation sites is due to the action of a modified "oligosaccharyltransferase" [41].

In analogy with yeast, phosphate residues were detected on N-glycans of some filamentous fungi. A small amount of the carbohydrate portion of β-galactosidase from *A. oryzae* was found to consist of short high-mannose glycans containing phosphate in monoester linkage and galactomannan-type sugar chains with phosphate in mono- and diester linkages [42]. The presence of phosphate residues was also established on N-glycans of cellobiohydrolase I secreted by the filamentous fungus *T. reesei* [40,43]. Using a combination of different techniques among which homonuclear and heteronuclear NMR techniques, the structure of ManPGlcMan7GlcNAc2 was proposed (Figure 2(A) [43,44]. At the moment, the linkage positions of phosphate residues on N-glycans of most filamentous fungi have not been studied in enough detail to speculate on substrate specificities and functions of phosphorylating enzymes. It may be possible that the phosphorylation process resembles that of *S. cerevisiae* which has been studied more extensively and generally appears to occur both on α-1,2 and on α-1,6 linked Man α-1,2-Man (Figure 2B) [45–48]. Next to phosphorylation of N-glycans, phosphorylation of O-glycans has been reported to occur in yeast [49].

Table 2. An overview of partially or completely characterised oligosaccharides on glycoproteins from filamentous fungi

Species	*Studied glycoprotein*	*Number of glycans*	*N-glycan structure*	*O-glycan structure*	*Presence of phosphate*	*Reference*
A. oryzae	Taka amylase A	1 N	$Man_6GlcNAc_2$	–		[85]
	Coprinus cinereus peroxidase	1 N / 2 O	$Man_{(1-7)}GlcNAc_2$ single GlcNAc	$Man_{(1-5)}$	–	[86]
	β-galactosidase	4 N	96 % of short sugarchains including $Man_{(5-11)}GlcNAc_2$ 4 % hyperglycosyl (galactomannan)	–	+	[42]
	Glucoamylase			$Man_{(1-3)}$ GlcMan		[62]
A.saitoi	Acid carboxypeptidase		$Man_{(5-6)}GlcNAc_2$ $Man_{(8-12)}GlcNAc_2$	Man		[66]
A. niger	Endo-polygalacturonase	1 N	$Man_{(5-11)}GlcNAc_2$	–	–	[57]
	Glucose-oxidase		$Man_{(5-7)}GlcNAc_2$ + non-characterised short sugarchains	Man		[56]
	α-D-glucosidase		$Man_{(8-9)}GlcNAc_2$ $GlcMan_9GlcNAc_2$ $GalfMan_{(5-8)}GlcNAc_2$			[51]
	α-D-galactosidase		$Man_{(5-6)}GlcNAc_2$ $Man_9GlcNAc_2$ $GlcMan_9GlcNAc_2$ $GalfMan_4GlcNAc_2$ $GalfMan_5GlcNAc_2$			[50]
	Phytase		$Man_{(5-10)}GlcNAc_2$			[58]
	Glucoamylase	2 N/ 35 O		$Man_{(1-3)}$ GlcMan		[62]
A. nidulans	Invertase		Galactomannan (hyperglycosylation?)	–	+(?)	[55]
A.tubigensis	PNGase At	9N/1O	$Man_{(5-9)}GlcNAc_2$ single GlcNAc	$Man_{(1-2)}$		[38]
A. awamori	Glucoamylase	2 N/ 40 O	NB	$Man_{(1-2)}$ $GlcMan_2$ $GalMan_2$		[69]
Var. *Kawachi*	Glucoamylase		Galactomannaan	Glucosylated high-mannose glycans		[54]
	Glucoamylase		$Man_2GlcNAc_2$			[33]
T. reesei	Cellobiohydrolase I	3 N/ 8 O	$Man_5GlcNAc_2$ $Man_9GlcNAc_2$	$Man_{(1-4)}$		[59]
	Cellobiohydrolase I		single GlcNAc $Hex_{(5-11)}HexNAc_2$	$Man_{(1-3)}$ Sulphate addition	+	[40]
	Cellobiohydrolase I		single GlcNAc			[39]
	Cellobiohydrolase I		$Man_{(5-7)}GlcNAc_2$ $Glc(ManP)Man_7GlcNAc_2$		+	[43]
T. viride	Cellobiohydrolase C	? N/17 O	single GlcNac	$Man_{(1-5)}$ GlcMan GalMan		[61]

– =absent; + =present
Empty boxes indicate that data are lacking.

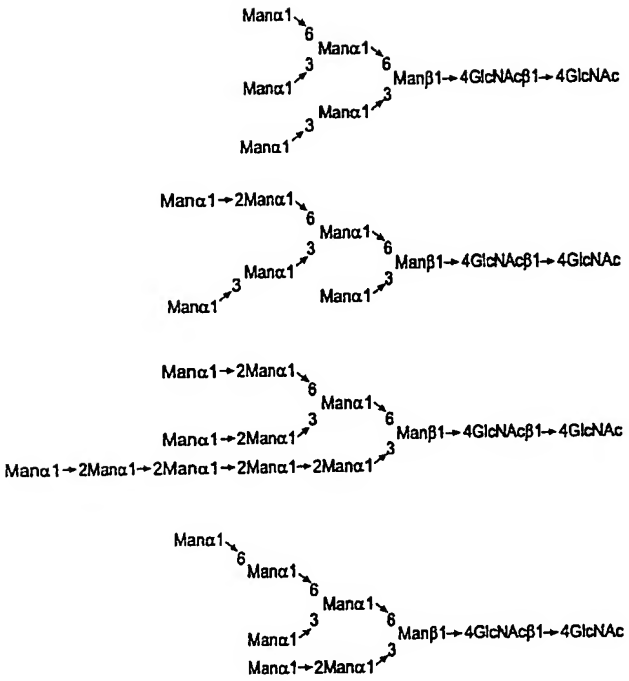


Figure 1. Fungi-type N-glycan structures. Mannose residues in red are probably incorporated by not yet characterised mannosyltransferases. The two upper oligosaccharides were found on glucose oxidase from *A. niger*, the third on acid carboxypeptidase from *A. saitoi* and the fourth glycan on β-galactosidase from *A. oryzae*.

Glucose and galactose residues have been detected at the non-reducing end of N-glycans of several species of filamentous fungi. Alfa-1,2-linked glucoses were found which probably resulted from inefficient trimming by glucosidase II. This lack of trimming has been observed with several species, and hence, it is a question whether this could be characteristic of filamentous fungi [43,51,52]. The presence of galactose residues was deduced from analyses of the monosaccharide compositions of N-glycans, but for some time it was difficult to assign their linkage positions. Groisman et al. [52] demonstrated that *Ascobolus furfuraceus* synthesised galactoses in the furanose form and not in the usual pyranose form. Alfa-linked galactofuranoses were detected on α-D-glucosidase and α-D-galactosidase from *A. niger* [50,51]. Beta-linked galactofuranose was detected on ascorbate oxidase from Acremonium sp. H1-25 [53]. The structures of these glycans were resolved by NMR analyses and are presented in Figure 3. Other research groups also mentioned galactomannans on glycoproteins from filamentous fungi, but without adding structural information [54,55]. The presence of these galactomannans seems to be strain dependent since other *A. niger* strains apparently do not transfer galactoses to their glycans [56–58]. Significant strain dependent differences were also revealed in N-glycan structures of *T. reesei* [39,40,43,59]. Growth conditions have been found to influence the glycan modification of some proteins, possibly by modulating the expression of enzymes involved in the fungal glycosylation pathway [60]. For these reasons it is important to mention the fungal strain and the exact growth conditions when glycan structures of a specific glycoprotein are reported.

The O-glycans found in filamentous fungi up till now differ from those found in higher eukaryotes [32]. Filamentous fungi synthesise O-glycans that vary from a single mannose to linear mannopentaoses. In some cases, the latter glycans were found to be substituted with glucose and/or galactose [40,61–63]. Harrisson et al. [40] suggested the presence of sulfate groups on O-glycans from *T. reesei*.

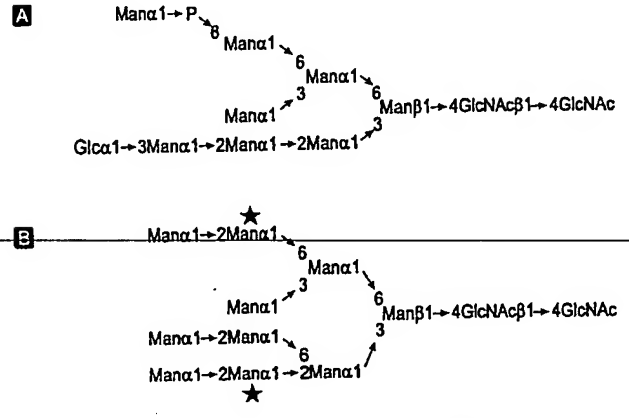
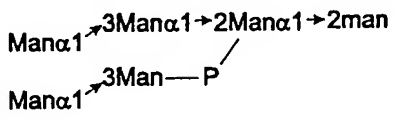


Figure 2A. The structure of a phosphorylated N-glycan on *T. reesei* Rut-C30 cellobiohydrolase I. 2B Core phosphorylation on *Saccharomyces cerevisiae* glycoproteins. The positions where phosphate groups are added to the core portion of yeast N-glycans are marked with asterisks.



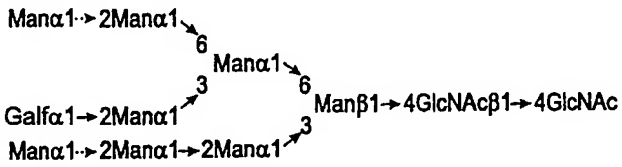


Figure 3. Structures of galactosylated fungal N-glycans. The above N-glycans were found on α-D-glucosidase from *A. niger*. The lower N-glycan structure was found on ascorbate oxidase from *Acremonium* sp. H1-25.

The importance of glycosylation for properties of homologous and heterologous proteins

Analogous to the mammalian system, protein-linked glycans are important for the biological activity of some fungal proteins. In most cases observed sofar, however, N-glycans do not seem to be very important for the biochemical properties of fungal proteins, although much still has to be learned. In order to illustrate the above statements, some examples are given.

The zygomycete fungus *Rhizomucor pusillus* secretes aspartic proteinase, an enzyme which is used as a milk coagulant in cheese factories [64]. Production of this proteinase as a heterologous protein in *A. oryzae* resulted in increased mannosylation of the proteinase compared to the natural product. The recombinant proteinase showed a higher proteolytic activity and appeared to be less effective in milk-clotting. Deglycosylation by glycosidase or by amino acid replacement again led to a decrease of the first activity and an increase of the latter [65], indicating a functional importance of the glycan composition for the biological activity of this proteinase.

Aspergillus saitoi is known to secrete several extracellular proteinases, such as acid carboxypeptidase. Chiba et al. [66] showed that no extracellular carboxypeptidase could be found after tunicamycin treatment of *A. saitoi* cells, from which they concluded that N-glycosylation is important for secretion of this enzyme. Glycosylation may be also important for the secretion of heterologous proteins in fungi. In some cases the yield of heterologous protein produced in filamentous fungi is very low, which could be due (partly) to ineffective glycosylation. Morkeberg et al. [67] reported that overproduction of α-amylase in *Aspergillus oryzae* resulted in non-glycosylated product. An explanation could be that the glycosylation system cannot catch up with the high production rate. With α-amylase the glycosylation deficiency did not affect the production rate. For some heterologous proteins, however, glycosylation may be essential to obtain the proper conformation and high secretion levels. For example, the production of tissue plasminogen activator (tpA) as a well secreted product in Trichoderma has not been successful until now. Jarvis and Summers [68] demonstrated that secretion of tPA from insect cells is impaired when N-glycosylation is prevented. Similarly, inefficient glycosylation could be one of the factors responsible for the inefficient production of tPA in Trichoderma.

Neustroev et al. [69] showed that trimming of the O-glycans of *A. awamori* glucoamylase with α-mannosidase resulted in a decreased thermal and pH stability of the protein, indicating that O-glycosylation can confer thermostability to a protein. Goto et al. [36] described expression of a hyperglycosylated form of glucoamylase in *A. awamori var. kawachi*. This modified glucoamylase possessed higher digestibility of raw starch and higher stabilities in response to heat and extreme pH. Goto et al. [63] showed that O-glycans are involved in digestion by glucoamylase of raw starch through interaction with water, suggesting that O-glycans can be involved in hydration of substrate molecules. In a 'water-cluster-dissociating model' they proposed the oligomannose sugar chains to dissociate water clusters so that hydration of starch micelles with activated water molecules became possible. Partial replacement of mannose residues by glucoses led to a significantly decreased digestion of raw starch.

Absence of glycans can result in exposure of peptide fragments of a protein that are otherwise masked. As a result of this, aggregation can occur, the heterologous protein can be 'trapped' by interaction with an intracellular fungal protein or the protein is accessible to proteases and may be degraded [70,71].

Engineering of the glycosylation pathway in filamentous fungi

The N-glycans which are added to secreted proteins in filamentous fungi are dissimilar to those that are synthesised in mammalian cells. At the moment this is an important drawback for the application of fungi derived glycoproteins for therapeutic use [43,72,73]. Since compared to higher eukaryotes filamentous fungi are very easy to genetically modify, it is feasible to construct "lack of function" mutants that are disturbed in specific unwanted glycosylation steps, provided that the glycosylation pathway and the genes encoding the enzymes involved, are known. In contrast to yeasts, filamentous fungi are capable of extensive trimming of their N-glycans. A few fungal strains have been found that preferentially form Man5GlcNAc2 glycans [Maras and Contreras, unpublished results]. Furthermore, by selection of specific strains, in combination with mutagenesis it seems feasible to obtain strains that do not include phosphate groups in their glycans, and have lost the ability to hyperglycosylate, but instead mainly synthesise the Man5GlcNAc2 glycan that is the preferred starting glycan for complex-type glycosylation [74]. Subsequent engineering steps, i.e., introduction of the required mammalian glycosyltransferases may theoretically lead to strains that are able to accomplish complex-type glycosylation. The latter steps will be more complicated to accomplish, however, as the expressed heterologous glycosyltransferases have to be localised in specific membrane compartments, their activity levels should be adequate, and the donor substrates should be available in the fungi. Especially the last step, i.e. sialylation might prove difficult, as up till now no evidence is present that the donor substrate, CMP-neuraminic acid, is present in fungi. However, it may be possible to add *in vitro* sialic acid to recombinant glycoproteins by enzymatic synthesis. Recently it has been demonstrated that at least the first step towards a complex-type glycosylation, i.e. introduction of mammalian N-acetylglucosaminyltransferase I (GlcNAc-

T1) in filamentous fungi, has been accomplished. The cDNA encoding rabbit GlcNAc-TI has been introduced in the genome of *A. nidulans*. Kalsner *et al.* [75] demonstrated production of active enzyme by the fungus, but no evidence was presented for the *in vivo* transfer of GlcNAc residues to fungal glycans. In *T. reesei*, however, this *in vivo* transfer step has indeed been demonstrated. Structural elucidation by NMR of N-glycans of a strain, transformed with the cDNA encoding human GlcNAc-TI, established the formation of GlcNAcMan5GlcNAc2 in *T. reesei* [Maras et al., manuscript in preparation]. This result emphasises the fact that conversion of the glycosylation synthesis pathway is possible. However, the efficiency is not yet optimal and needs to be improved.

To take advantage of the high capacity of filamentous fungi for protein secretion, the glycosylation process should not only lead to correct mammalian-like glycans, but should also be stimulated to an elevated level to keep up with the high protein production to provide fully glycosylated and secreted glycoproteins.

Until now, synthesis of N- and O-glycans in filamentous fungi is very poorly studied. Since so little is known about glycan processing in filamentous fungi, the well studied pathway of the yeast *S. cerevisiae* is often used as a reference [76]. In fungi, only some steps of the dolichol-phosphate cycle for synthesis of the common Glc3Man9GlcNAc2 precursor glycan have been studied. Probably, this part of the N-glycosylation pathway is analogous to that in yeast. Palamarczyk et al. [32] mentioned that glycosylation can be enhanced by making available more of the lipid intermediates of the 'dolichol phosphate cycle'. Raising the temperature for growth of fungal cells for instance influences the dolichol content in ER membranes [77]. At elevated temperatures, dolichol kinase activity is stimulated. By adding Tween 80 or choline to the growth medium, an *in vivo* enhancement of mannose phosphate dolichol synthase (= MPD synthase) activity has been observed.

Some genes of this 'dolichol-phosphate cycle' have been cloned, amongst them dolichol phosphoryl mannose synthase from *Ustilago maydis* [78] and GTP:α-D-mannose-1-phosphate guanyltransferase from *T. reesei* [32]. Kruszewska *et al.* [79] overexpressed *S. cerevisiae* MPD synthase in Trichoderma and demonstrated enhanced cellulase secretion. It is known that in higher eucaryotes, terminal galactose residues on glycoproteins are recognized by the asialoglycoprotein receptor, resulting in the removal of these proteins from the circulation [80]. To convert fungi-like glycans to sialylated glycans, some "shortcuts" are proposed, which result in non-natural glycoproteins. Berg et al. [81] suggest to remove *in vitro* the major part of the glycan trees using an endo-N-acetylglucosaminidase. Depending on the enzyme used (EndoH or EndoF), the innermost one or two core GlcNAc residues are left on the protein. These residues are the acceptor for the successive action of a β-1,4[3]-galactosyltransferase and a sialytransferase, resulting in a typical sialylated lactosamine outer-chain. The endo-glycanase reaction is performed on native proteins. However, in such conditions not all glycans may be accessible to the enzyme and thus, some recognition determinants for mammalian receptors may still be present.

Conclusions

Filamentous fungi offer a very attractive, safe and cheap expression system for high level production of proteins. In contrast to yeast, they have a well developed secretion pathway, and naturally secrete high levels of proteins. At this moment, the therapeutic use of glycoproteins produced in filamentous fungi is very limited, as the glycans formed are dissimilar from mammalian glycans. However, specific filamentous fungal strains are available that can produce glycoproteins with short oligo mannose-type glycans. More knowledge and insight in the glycosylation pathway is needed to be able to engineer the glycosylation pathway to mimick a mammalian type of glycosylation. The prospects, however, to succesfully accomplish such an engineering by combined "lack of function" mutagenesis to prevent unwanted glycan epitopes and a "gain of function" engineering to add specific monosacharides to the glycan, are good.

Acknowledgments

We wish to thank Drs. Wouter Laroy and Steven Geysens for their contribution to this paper.

References

1 Varki A (1993) *Glycobiology* **3**: 97–130.
2 Cumming DA (1991) *Glycobiology* **1**: 115–30.
3 Olijve W, de Boer W, Mulders JW, Van Wezenbeek PM (1996) *Mol Human Reprod* **2**: 371–82.
4 Sasaki H, Bothner B, Dell A, Fukuda M (1987) *J Biol Chem* **262**: 12059–76.
5 Altmann F (1997) *Glycoconj J* **14**: 643–46.
6 Hsu TA, Takahashi N, Tsukamoto Y, Kato K, Shimada I, Masuda K, Whiteley EM, Fan JQ, Lee YC, Betenbaugh MJ (1997) *J Biol Chem* **272**: 9062–70.
7 Davidson DJ, Castellino FJ (1991) *Biochemistry* **30**: 6167–74.
8 Davidson DJ, Castellino FJ (1991) *Biochemistry* **30**: 6689–96.
9 Davidson DJ, Fraser MJ, Castellino FJ (1990) *Biochemistry* **29**: 5584–90.
10 Tucker GA, Woods LFJ, eds (1995) *Enzymes in Food Processing* (2nd ed). London: Blackie.
11 Finkelstein DB, Rambosek J, Crawford MS, Soliday CL, Mc Ada PC, Leach J (1989) In *Genetics and Molecular Biology of Industrial Microorganisms* (Hershberger Cl, Queener SW, Hegemann G, eds) pp 295–300 Washington DC: American Society for Microbiology,

12 Verdoes, JC, Punt PJ, Van den Hondel CAMJJ (1995) *Appl Microbiol Biotechnol* **43:** 195–205.

13 Jeenes DJ, Mackenzie DA, Roberts IN, Archer DB (1991) *Biotechn Genet Eng Rev* **9:** 327–67.

14 Van den Hondel CAMJJ, Punt PJ, van Gorcom RFM (1991) In *More Gene Manipulation in Fungi* (Bennett JW, Lasure LL, eds), p 396. New York: Academic Press.

15 Gwynne DJ, Devchand M (1992). In *Aspergillus, the Biology and Industrial Application* (Bennet JW, Klich MA, eds) pp 203–14. London: Butterworth.

16 Gouka RJ, Punt PJ, Van den Hondel CAMJJ (1997) *Appl and Environmental Microbiol* **63:** 488–97.

17 Gouka RJ, Punt PJ, Hessing JGM, Van den Hondel CAMJJ (1996). *Appl Environ Microbiol* **62:** 195–205.

18 Nyyssonen E, Keranen S (1995) *Curr Genet* **28:** 71–9.

19 Gouka RJ, Punt PJ, Van den Hondel CAMJJ (1997) *Appl Microbiol Biotechnol* **47:** 1–11.

20 Broekhuijsen MP, Mattern IE, Contreras R, Kinghorn JR, Van den Hondel CAMJJ (1993) *J Biotech* **31:** 135–45.

21 Van Hartingsveldt W, Van Zeijl CMJ, Veenstra AE, Van den Berg JA, Pouwels PH, Van Gorcom RFM, Van den Hondel CAMJJ (1990) In *Proceedings of 6th International Symposium on Genetics of Industrial Microorganisms* (Heslot H, Davies J, Florent J, Bibichon L, Durant G, Penasse L, eds), p 107, Société Française de Microbiologie.

22 Roberts IN, Jeenes DJ, MacKenzie DA, Wilkinson AP, Summer IG, Archer DB (1992) *Gene* **122:** 155–61.

23 Archer DB, Peberdy JF (1997) *Crit Rev Biotechn* **17:** 273–306.

24 Dunn-Coleman NS, Bloebaum P, Berka RM, Bodie E, Robinson N, Armstrong G, Ward M, Przetak M, Carter GL, LaCost R, Wilson Lj, Kodama KH, Baliu EF, Bower B, Lamsa M, Heinsohn H (1991) *Biotechnology* **9:** 976–81.

25 Ward PP, Piddington CS, Cunningham GA, Zhou X, Wyatt RD, Conneely OM (1995) *Biotechnology* **13:** 498–503.

26 Civas A, Eberhard R, Le Dizet P, Petek F (1984) *Biochem J* **219:** 857–63.

27 Kobata A, Amano J (1987) *Meth Enzymol* **138:** 779–85.

28 Gaikwad SM, Keskar SS, Khan MI (1995) *Biochim Biophys Acta* **1250:** 144–48.

29 Eades CJ, Gilbert A, Goodman CD, Hintz WE (1998) *Glycobiology* **8:** 17–33.

30 Luonteri E, Tenkanen M, Viikari L (1998) *Enzyme Microb Technol* **22:** 192–98.

31 Eneyskaya EV, Kulminskaya AA, Savel'ev AN, Shabalin KA, Golubev AM, Neustroev KN (1998) *Biochem Biophys Res Commun* **245:** 43–9.

32 Palamarczyk G, Maras M, Contreras R and Kruzewska J (1998) *In Trichoderma and Gliocladium. Enzymes, Biological Control and Commercial Applications* (Harman GE, Kubicek CP, eds) vol. 1, pp 121–33. London: Taylor and Francis Ltd

33 Aleshin AE, Hoffman C, Firsov LM, Honzatko RB (1994) *J Mol Biol* **238:** 575–91.

34 Yoshida T, Ichishima E (1995) *Biochim Biophys Acta* **1263:** 159–162.

35 Inoue T, Yoshida T, Ichishima E (1995) *Biochim Biophys Acta* **1253:** 141–45.

36 Goto M, Ekino K, Furukawa K (1997) *Appl Envir Microbiol* **63:** 2940–43.

37 Devchand M, Gwynne DI (1991) *J Biotechnology* **17:** 3–10.

38 Ftouhi-Paquin N, Hauer CR, Stack RF, Tarentino AL, Plummer TH Jr (1997) *J Biol Chem* **272:** 22960–65.

39 Klarskov K, Piens K, Stahlberg J, Hoj PB, Beeumen JV, Claeyssens M (1997) *Carbohydr Res* **304:** 143–54.

40 Harrison MJ, Nouwens AS, Jardine DR, Zachara NE, Gooley AA, Nevalainen H, Packer NH (1998) *Eur J Biochem* **256:** 119–27.

41 Silberstein S, Gilmore R (1996) *FASEB J* **10:** 849–58.

42 Nakao Y, Kozutsumi Y, Funakoshi I, Kawasaki T, Yamashina I, Mutsaers JHGM, Van Halbeek H, Vliegenthart JFG (1987) *J Biochem* **102:** 171–79.

43 Maras M, De Bruyn A, Schraml J, Herdewijn P, Claeyssens M, Fiers W, Contreras R (1997) *Eur J Biochem* **245:** 617–25.

44 De Bruyn A, Maras M, Schraml J, Herdewijn P, Contreras R (1997) *FEBS Lett* **405:** 111–13.

45 Hashimoto C, Cohen RE, Zhang WJ, Ballou CE (1981) *Proc Natl Acad Sci USA* **78:** 2244–48.

46 Hernandez LM, Ballou L, Alvarado E, Tsai P, Ballou CE (1989) *J Biol Chem* **264:** 13648–59.

47 Ballou L, Hernandez LM, Alvarado E, Ballou CE (1990) *Proc Natl Acad Sci USA* **87:** 3368–72.

48 Hernandez LM, Olivero I, Alvarado E, Larriba G (1992) *Biochemistry* **31:** 9823–31.

49 Jars MU, Osborn S, Forstrom J, MacKay VL (1995) *J Biol Chem* **270:** 24810–17.

50 Takayanagi T, Kushida K, Idonuma K, Ajisaka K (1992) *Glycoconj J* **9:** 229–34.

51 Takayanagi T, Kimura A, Chiba S, Ajisaka K (1994) *Carbohydrate Res* **256:** 149–58.

52 Groisman JF, de Lederkremer RM (1987) *Eur J Biochem* **165:** 327–32.

53 Ohta M, Emi S, Iwamoto H, Hirose J, Hiromi K, Itoh H, Shin T, Murao S, Matsuura F (1996) *Biosci Biotech Biochem* **60:** 1123–30.

54 Goto M, Kuwano E, Kanlayakrit W, Hayashida S (1995) *Biosci Biotechnol Biochem* **1:** 16–20.

55 Chen J, Saxton J, Hemming FW, Peberdy JF (1996) *Biochim Biophys Acta* **1296:** 207–18.

56 Takegawa K, Kondo A, Iwamoto H, Fujiwara K, Hosokawa Y, Kato I, Hiromi K, Iwahara S (1991) *Biochem Int* **25:** 181–90.

57 Yang Y, Bergmann C, Benen J, Orlando R (1997) *Rapid Comm Mass Spectr* **11:** 1257–62.

58 Panchal T, Wodzinski RJ (1998) *Prep Biochem Biotech* **28:** 201–7.

59 Salovuori I, Makarow M, Rauvala H, Knowles J, Kttritinen L (1987) *Biotechnology* **5:** 152–56.

60 Goochee CF, Monica T (1990) *Biotechnology* **8:** 421–27.

61 Gum EK Jr, Brown RD Jr (1976) *Biochim Biophys Acta* **446:** 371–86.

62 Gunnarsson A, Svensson B, Nilsson B, Svensson S (1984) *Eur J Biochem* **145:** 463–67.

63 Goto M, Kuwano E, Kanlayakrit W, Hayashida S (1995) *Biosci Biotech Biochem* **59:** 16–20.

64 Murakami K, Takeuchi K, Beppu T, Horinouchi S (1998) *Microbiology* **144:** 1369–74.

65 Murakami K, Aikawa J, Horinouchi S, Beppu T (1993) *Mol Gen Genet* **241:** 312–18.

66 Chiba Y, Yamagata Y, Iijima S, Nakajima T, Ichishima E (1993) *Curr Microbiol* **27:** 281–88.

67 Morkeberg R, Carlsen M, Nielsen J (1995) *Microbiology* **141:** 2449–54.

68 Jarvis DL, Summers MD (1989) *Mol Cell Biol* **9:** 214–23.
69 Neustroev KN, Golubev AM, Firsov LM, Ibatullin FM, Protasevich II, Makarov AA (1993) *FEBS Lett* **316:** 157–60.
70 Opdenakker G, Rudd PM, Ponting CP, Dwek RA (1993) *FASEB J* **7:** 1330–37.
71 Wang C, Eufemi M, Turano C, Giartosio A (1996) *Biochemistry* **35:** 7299–307.
72 Jenkins N, Parekh RB, James DC (1996) *Nature Biotechnology* **14:** 975–81.
73 Maras M, Saelens X, Laroy W, Piens K, Claeyssens M, Fiers W, Contreras R (1997) *Eur J Biochem* **249:** 701–7.
74 Vella GJ, Paulsen H, Schachter H (1983) *Can J Biochem Cell Biol* **62:** 409–17.
75 Kalsner I, Hintz W, Reid LS, Schachter H (1995) *Glycoconjugate J* **12:** 360–70.
76 Herscovics A, Orlean P (1993) *FASEB J* **7:** 540–50.
77 Kruszewska J, Kubicek CP, Palamarczyk G (1994) *Acta Biochim Pol* **41:** 331–37.
78 Zimmerman JW, Specht CA, Xoconostle Cazares B, Robbins PW (1996) *Yeast* **12:** 765–71.
79 Kruszewska JS, Saloheimo M, Penttila M, Palamarczyk G (1998) *Curr Genet* **33:** 445–50.
80 Ashwell G, Harford J (1982) *Annu Rev Biochem* **51:** 531–54.
81 Berg EL, Robinson MK, Mansson O, Butcher EC, Magnani JL (1991) *J Biol Chem* **266:** 14869–72.
82 Nyyssonen E, Penttila M, Harkki A, Saloheimo A, Knowles JK, Keranen S (1993) *Biotechnology* **11:** 591–95.
83 Frenken LG, Hessing JG, Van den Hondel CA, Verrips CT (1998) *Res Immunol* **149:** 589–99.
84 Honda G, Matsuda A, Zushi M, Yamamoto S, Komatsu K (1997) *Biosci Biotech Biochem* **61:** 948–55.
85 Yamaguchi H, Ikenaka T, Matsushima Y (1971) *J Biochem* **70:** 587–94.
86 Limongi P, Kjalke M, Vind J, Tams JW, Johansson T, Welinder, KG (1995) *Eur J Biochem* **227:** 270–76.

Accepted 18 March 1999.